SUPPLEMENTARY MATERIAL FOR:

Two Pathways for Cyclooxygenase-2 Protein Degradation *In Vivo*

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Running Title: Cyclooxygenase Knock-in Mouse

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Supplementary Materials and Methods

Preparation of Mutant Murine COX-2 cDNAs. Murine COX-2 cDNA (1) was inserted into pCDNA5/FRT/TO (Invitrogen). COX-2 mutants including N67A, N144A, N410A, N594A and N410A + N594A mouse were prepared using a Stratagene site-directed mutagenesis kit using the subcloned vector, pCDNA5/muCOX-2wt as a template and appropriate oligonucleotide primers: Asn-67 to Ala, 5'-ggattctatggtgaaGCctgtactacacctg-3' and 5'-caggtgtagtacagGCttcaccatagaatcc-3'; Asn-144 to Ala, 5'-ggaagccttctccGCcctctcctac-3' and 5'-gtaggagaggGCggagaaggcttcc-3'; Asn-410 to Ala, 5'-cagtttctctacGCcaactccatcctcc-3' and 5'-ggaggatggagttgGCgtagagaaactg-3'; and Asn-594 to Ala, 5'-cagccaccatcGCTgcaagtgcctc-3' and 5'-gaggcacttgcAGCgatggtggctg-3'.

Generation of Cell Lines Expressing Native or Mutant COX-2. Flp-In^{TM} 293 cells were plated in 35 mm culture dish and transfected with native or mutant mouse COX-2/ pCDNA5, pOG44 and LipofectAMINE 2000 according to the manufacturer's instruction. The positive colonies were selected using cyclooxygenase assays (2) using cell lysates and immunoblotting essentially as described previously.

Expression of COX-2 proteins in Flp-In 293 cells. Expression of native or mutant COX-2 proteins were induced after a 24 h serum starvation in DMEM medium containing 0.2 % HFBS and by incubation with DMEM containing 10 % HFBS, 10 μg/ml tetracycline with or without 25 μM kifunensin (KIF). The cells were harvested at different time points and were stored at -80^{o} C until use.

SDS-PAGE and Western Blotting**.** Frozen cell pellets were lysed in 20 mM TrisHCl, pH 7.4, containing 150 mM NaCl, 2 mM EDTA, 1% NP-40, 50 mM NaF, 10% glycerol and a cocktail of protease inhibitors (Roche) for 10 min on ice. Insoluble material was removed by

centrifugation at 16,000 x g at 4° C for 20 min. Protein concentrations were determined using a Pierce BCA protein assay kit. Proteins were separated by electrophoresis on 4-12% polyacrylamide gradient gels or 7% Tris-acetate polyacrylamide gels (Invitorogen). For immunoblotting, the proteins were electroblotted onto a polyvinylidene fluoride (PVDF) membrane with a semi-dry blotter (Bio-Rad). The membranes were washed (blocked) overnight in 25 mM TrisHCl, pH7.4, containing 0.8% NaCl, 0.02% KCl, 0.1% Tween 20 (TBS-T), and 5% skim milk. The membranes were then incubated with appropriate antibodies against COX-1 (Cayman Chemical Co.), COX-2 or actin for 2 h. After the membrane had been rinsed three times for ten min with TBS-T containing 1% skim milk, it was incubated with goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) for 1h. After washing four times for ten min with TBS-T, immunodetection was performed using a Pierce SuperSignal West Pico Chemiluminescent Substrate Kit followed by exposure to X-ray film.

Deglycosylation of COX-2 Protein. For deglycosylation reactions, protein samples were boiled in NuPAGE SDS sample loading buffer with dithiothreitol and then incubated with Endo H for 3 h at 37° C (2,3).

Hematoxylin and Eosin Staining of Mouse Kidneys. Female COX-2 null mice were obtained from Taconic Farms. Renal sections from twelve week old mice were prepared and stained essentially as described by Morham *et al.* (4).

Fig. S-1. N-glycosylation of wild type (WT) and mutant murine (mu) COX-2. ***A.*** Domain structures and consensus N-glycosylation sites of COX-1 and COX-2. *EGF*, epidermal growth factor; *MBD*, membrane binding domain; *ER*, endoplasmic reticulum. ***B.*** Expression of WT (Wild Type) muCOX-2 in HEK293 cells stably expressing the enzyme following treatment of the cells with tetracycline for the indicated times in the presence or absence of kifunensine (KIF). Methods for preparing stably expressing muCOX-2 are indicated above and are essentially the same as those reported previously for human COX-2 (3). The cells were harvested and cell lysate protein (20 μg/lane) was subjected to western blot analysis with anti-mouse COX-2 (αGln583-Asn594) and anti-actin antibodies as described in the Materials and Methods. ***C.*** Expression of muCOX-2 mutants in HEK293 cells stably expressing WT or various muCOX-2 mutants following treatment with tetracycline for 24 hr in the presence or absence of kifunensine (KIF). Cell lysate protein (20 μg/lane) was subjected to western blot analysis as described above. WT (***Lane 1***); N67A mutant (***Lane 2***); N144A mutant (***Lane 3***); N410A mutant (***Lane 4***); N594A mutant (***Lane 5***) and N410A + N594A mutant (***Lane 6***) muCOX-2. An aliquot of each protein was also treated with endoglycosidase H (Endo H) to remove N-linked mannose-containing glycosyl groups.

The results are consistent with those reported previously with huCOX-2 mutants (3) suggesting that Asn594 is N-glycosylated which, in turn, permits entry of COX-2 into the ERAD pathway. A second, higher molecular mass protein band appears when WT muCOX-2 or COX-2 mutants having an intact Asn594 are expressed in the presence of KIF. KIF inhibits ERAD by interfering with the processing of N-glycosylated proteins. As expected, treatment of WT and mutant proteins with Endo H to remove N-linked carbohydrate yielded proteins of the same molecular mass.

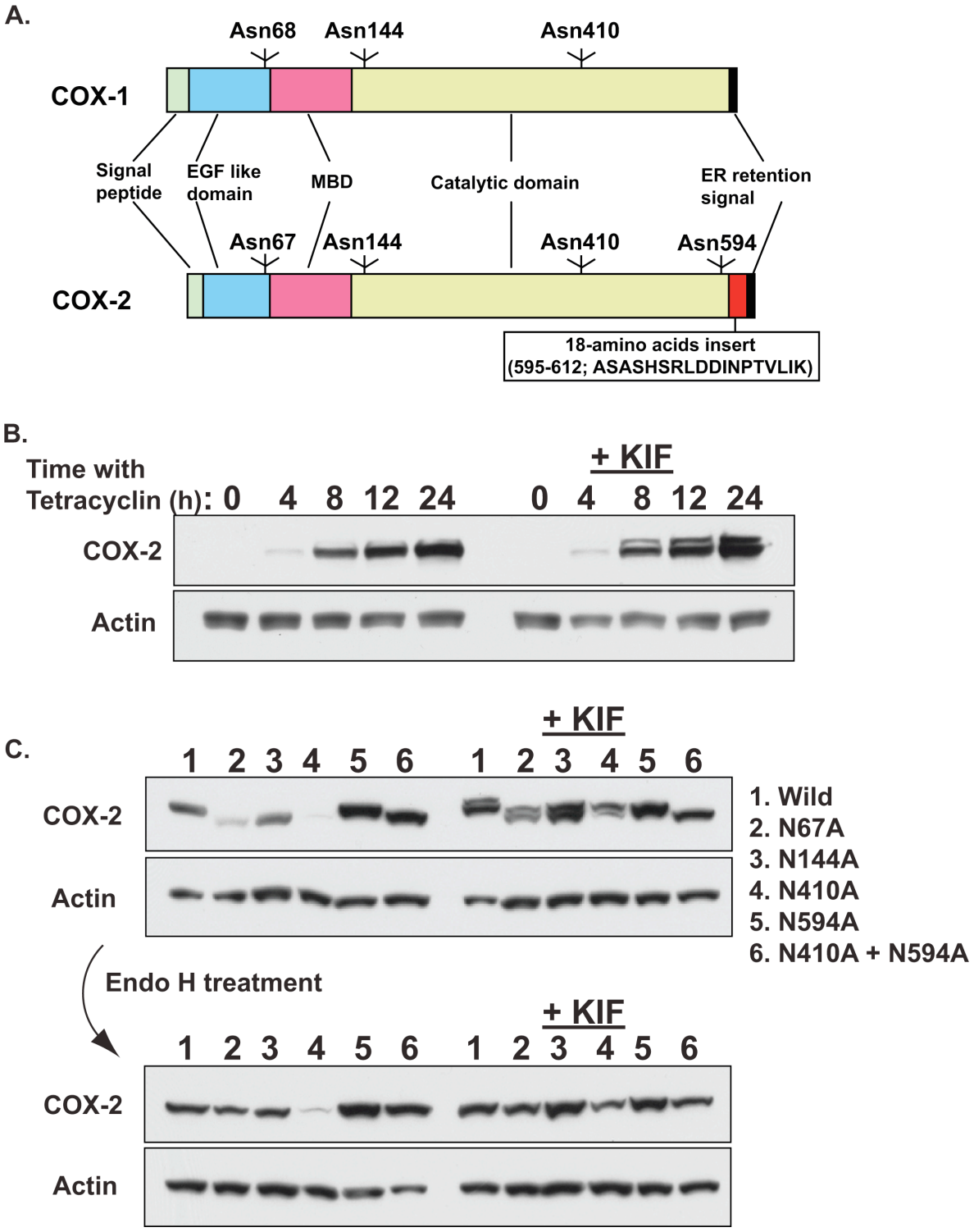
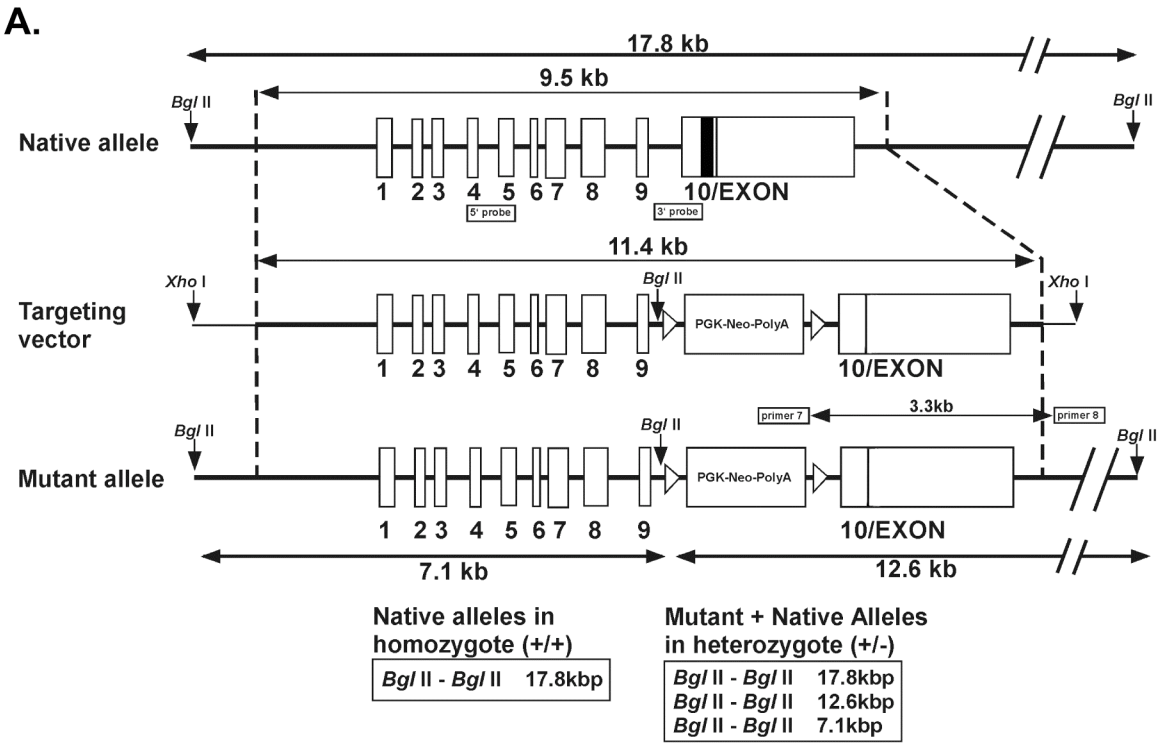

A.
Asn68
Asn144
Asn410
COX-1
Signal peptide
EGF like domain
MBD
Catalytic domain
ER retention signal
Asn67
Asn144
Asn410
Asn594
COX-2
18-amino acids insert
(595-612; ASASHSRLDDINPTVLIK)
B.
Time with Tetracyclin (h):
0 4 8 12 24
+ KIF
0 4 8 12 24
COX-2
Actin
C.
+ KIF
1 2 3 4 5 6 1 2 3 4 5 6
COX-2
Actin
1. Wild
2. N67A
3. N144A
4. N410A
5. N594A
6. N410A + N594A
Endo H treatment
+ KIF
1 2 3 4 5 6 1 2 3 4 5 6
COX-2
Actin

Fig. S-1.

Fig. S-2. Southern blotting of genomic DNA isolated from mouse ES cells expressing alleles for WT muCOX-2 and Δ18 COX-2 containing the LoxP-PGK-Neo-PolyA cassette. ***A***. Restriction map showing the location and sizes of *Bgl* II-*Bgl* II fragments (in boxes below) expected for WT COX-2 and Δ18 muCOX-2 genes (in the case of the Δ18 COX-2 construct containing the LoxP-PGK-Neo-PolyA cassette). ***B***. Genomic DNA isolated from three different mouse ES cell clones was treated with *Bgl* II, subjected to electrophoresis on a 0.6% agarose gel and hybridized for 48 hr with the indicated ^{32}P-labeled 5'- or 3'- muCOX-2 specific probe. ***Lane 1,*** Positive control 9.5 kbp fragment (5 pg); ***Lane 2***, control ES cell DNA (7.5 μg); ***Lane 3***, WS2H7 ES cell DNA (10 μg); ***Lane 4***, WS2D2 ES cell DNA (10 μg); ***Lane 5,*** WS2G5 ES cell DNA (10 μg).

The results indicate that appropriately sized *Bgl* II restriction fragments were present for the three ES cell clones designated WS2H7, WS2D2 and WS2G5 (lanes 3-5) containing one copy of the WT muCOX-2 gene and one copy of the Δ18 muCOX-2 gene having the inserted LoxP PGK neo polyA cassette.


A.
17.8 kb
9.5 kb
Bgl II
Native allele
1 2 3 4 5 6 7 8 9 10/EXON
5' probe
3' probe
11.4 kb
Xho I
Targeting vector
PGK-Neo-PolyA
primer 7
3.3kb
primer 8
Mutant allele
7.1 kb
12.6 kb
Native alleles in homozygote (+/+)
Bgl II - Bgl II 17.8kbp
Mutant + Native Alleles in heterozygote (+/-)
Bgl II - Bgl II 17.8kbp
Bgl II - Bgl II 12.6kbp
Bgl II - Bgl II 7.1kbp

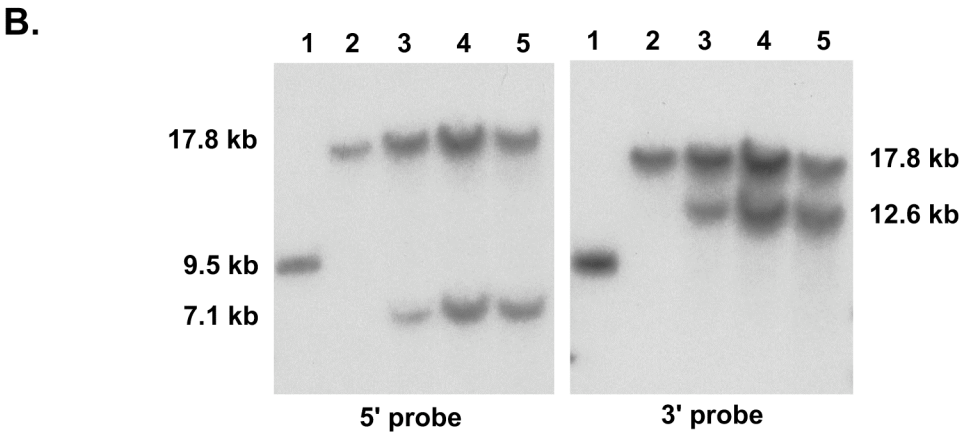

B.
1 2 3 4 5
17.8 kb
9.5 kb
7.1 kb
5' probe
3' probe
12.6 kb

Fig. S-2.

Table S-1. Comparison of Litter Sizes in Breeding of WT COX-2 and Δ18 COX-2 mice.

Breeder		Number of Litters	Litter size
Female	Male		
+/- [a]	+/-	16	6.6 ± 0.6 [b]
-/-	+/-	1	7
-/-	-/-	7	5.6 ± 1.1

[a] "+" indicates WT COX-2 gene and "-" indicates Δ18 COX-2 gene; nine different females and at least five different males were used in the +/- x +/- crosses; six different females and four different males were used in the -/- x -/- crosses. The animals used had been backcrossed at least three times into the C57BL/6J background.

[b] Values are number of animals ± SEM.

C57BL/6 mice have average litter sizes of 6.2 (http://members.cox.net/microinjectionworkshop/technical/breeding.html). Our results show that there are no obvious differences in litter sizes resulting from crosses of male or female mice carrying the Δ18 COX-2 gene and that the litter sizes are within the range expected for crosses between WT C57BL/6 mice.

Fig. S-3. Hematoxylin and Eosin staining of sections of renal cortex from WT, COX-2 null and Δ18 COX-2 mice. Kidneys were harvested from mice after euthanasia and fixed in 10% formalin in phosphate-buffered saline, pH 7.4. Kidneys were embedded in paraffin for staining with H & E. ***A***. Renal cortex from WT mouse. ***B***. Renal cortex from COX-2 null mouse. ***C.*** Renal cortex from Δ18 COX-2 mouse. (Original magnification, x 200) Arrows indicate glomeruli.

The results show that the renal cortex in COX-2 null mice exhibit glomerular hypoplasia and hypertrophy of glomeruli as reported previously (4), but sections from renal cortex of WT and Δ18 COX-2 mice are morphologically indistinguishable.

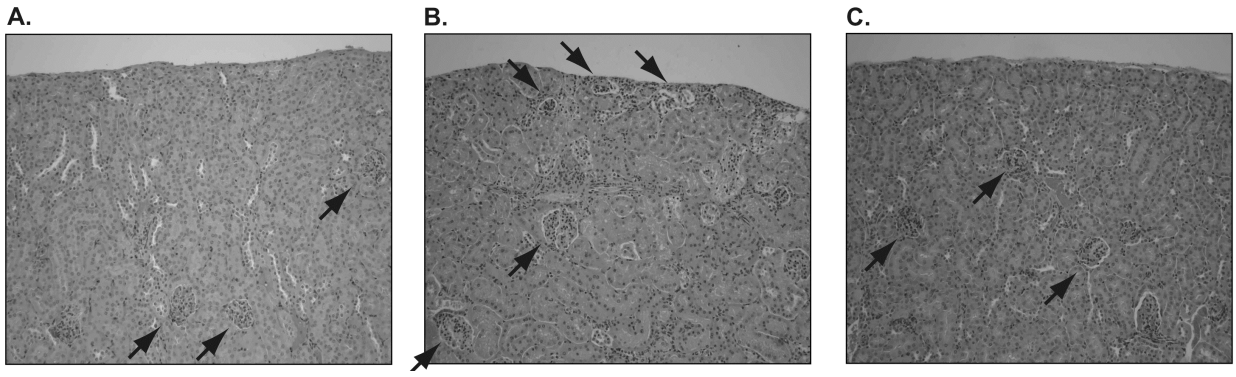


Fig. S-4.

Fig. S-4. Basal expression of COX-1 and COX-2 in tissues from WT and Δ18 COX-2 mice. Microsomal protein was prepared as described in Materials and Methods from homogenates of ***A.*** whole brain, ***B.*** heart, ***C.*** kidney and ***D.*** spleen obtained from 12 week old male or female WT or Δ18 COX-2 mice. Each number represents a different animal. The lanes with the same numbers in each panel represent tissue coming from the same animal. The same amounts of microsomal protein were applied in each lane; C, control WT muCOX-2 (0.05 μg) or control WT muCOX-1 (0.05 μg). The lane next to the control contained M.W. standards that are not visible.

The results indicate that at baseline Δ18 COX-2 mice express higher levels of immunoreactive COX-2 in brain that WT mice. However, COX-2 is not overexpressed in heart, kidney or spleen from Δ18 COX-2 mice.

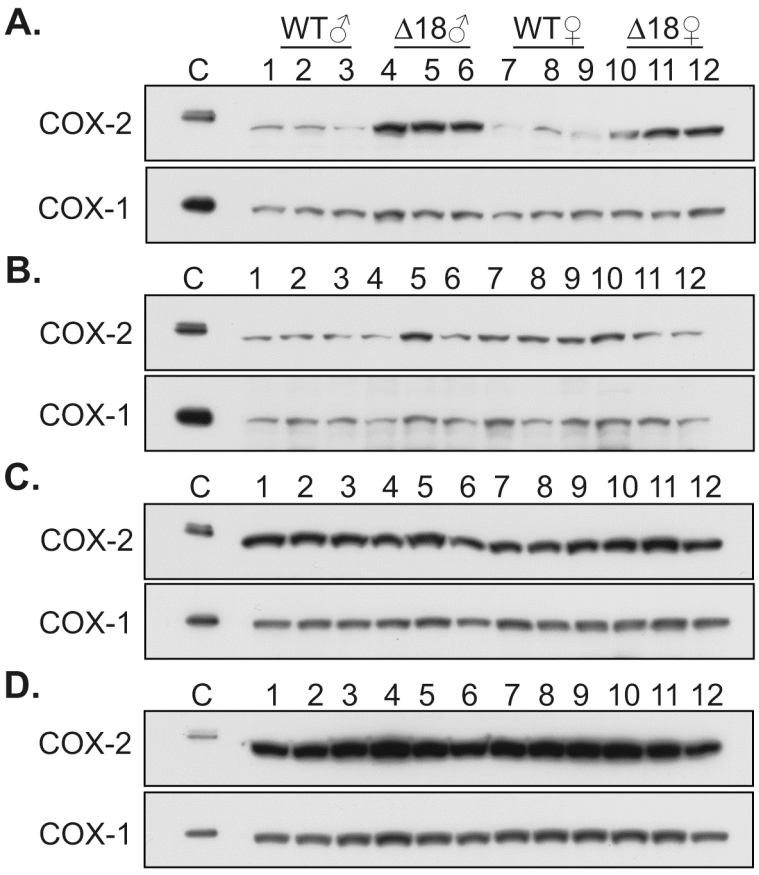


Fig. S-3.

References

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